Lantibiotics

International Edition: DOI: 10.1002/anie.201500927
German Edition: DOI: 10.1002/ange.201500927

Pinensins: The First Antifungal Lantibiotics

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Abstract: *Lantibiotics (lanthionine-containing antibiotics) from Gram-positive bacteria typically exhibit activity against Gram-positive bacteria. The activity and structure of pinensin A (**1**) and B (**2**), lantibiotics isolated from a native Gram-negative producer Chitinophaga pinensis are described. Surprisingly, the pinensins were found to be highly active against many filamentous fungi and yeasts but show only weak antibacterial activity. To the best of our knowledge, lantibiotic fungicides have not been described before. An in-depth bioinformatic analysis of the biosynthetic gene cluster established the ribosomal origin of these compounds and identified candidate genes encoding all of the enzymes required for post-translational modification. Additional encoded functions enabled us to build up a hypothesis for the biosynthesis, export, sensing, and import of this intriguing lantibiotic.*

Members of the genera *Chitinophaga* and *Flexibacter* are known producers of secondary metabolites with antimicrobial activity. However, the number of bioactive substances isolated from these species is very limited: The elansolids from *Chitinophaga* exhibit good activity against Gram-positive bacteria.[1] Furthermore, antibacterial compounds, as well as inhibitors of mammalian topoisomerase and elastase have been reported for different *Flexibacter* species.[2–5]

In our screening for bioactive secondary metabolites, strain *C. pinensis* DSM 28390 (= DSM 2588) exhibited strong antifungal activity. Through activity-guided fractionation, the antifungal activity could be assigned to two unknown peptides, pinensins A (**1**) and B (**2**). Herein we report the isolation, structure elucidation, biosynthetic origin, and antifungal activity of **1** and **2**.

The producer strain DSM 28390 was cultivated and compounds were isolated as described in the Supporting Information. The structures of both peptides were elucidated by using NMR spectroscopy and HPLC–HR-ESI-MS. Compound **1** has a monoisotopic mass of 2213.9622 Da with the molecular formula $C_{96}H_{139}N_{27}O_{30}S_2$, which was supported by the presence of $[M+3H]^{3+}$ and $[M+2H]^{2+}$ions.[6] Similarly, **2** has a monoisotopic mass of 2142.9251 Da and the molecular formula $C_{93}H_{134}N_{26}O_{29}S_2$, thus indicating one less alanine residue. Unsuccessful attempts at peptide sequencing or analysis of the ring topology by MS/MS, respectively, suggested overlapping intramolecular cyclization via thioethers.[7]

Consequently, 1D and 2D NMR spectroscopy, as well as Edman analysis, were used for the structure elucidation, which revealed **1** and **2** to be cyclic lantipeptides derived from 22 and 21 amino acids (aa), respectively, and incorporating two 3-methyl-lanthionine-bridged interwoven ring systems (Figure 1; see the Supporting Information for details).

In general, the absolute configurations of the asymmetric centers in Abu-S-Ala units of lantibiotics are reported as (2*S*,3*S*,6*R*)-3-methyl-lanthionine.[10] Exceptions were detected for cytolysin and haloduracin bearing methyllanthionine units with different stereochemical configuration in their A rings.[11] In the case of **1** and **2**, the usual (2*S*,3*S*,6*R*)-configuration was established by combining GC–MS analysis, Advanced Marfey's analysis, and a desulfurization approach (Figure 1 a and section 1.8.3 in Supporting Information).

Lantibiotics are regarded as ribosomally synthesized and post-translationally modified peptides, a rapidly growing category of natural products.[12] The first lantibiotic, nisin, was described as early as 1928.[13] The early lantibiotics were exclusively isolated from Gram-positive producers and they mainly act upon Gram-positive bacteria.[14] Through the use of genome mining, lantibiotic biosynthetic gene clusters have also been found in some Gram-negative bacteria.[15–17] Prochlorosin, a lantipeptide of Gram-negative origin that was heterologously produced in milligram quantities in *E. coli*, has been structurally characterized.[7] However, the pinensins reported herein could be isolated on a gram scale and can be considered the first lantibiotics isolated from a Gram-negative native producer.

Surprisingly, **1** and **2** were found to be only weakly active against bacteria (Table 1) but exhibited significant inhibition of fungi and yeasts in our initial screening. As a 1:1 mixture, they displayed broad antifungal activities with a minimum

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Supporting information for this article (experimental details) is available on the WWW under http://dx.doi.org/10.1002/anie.201500927.

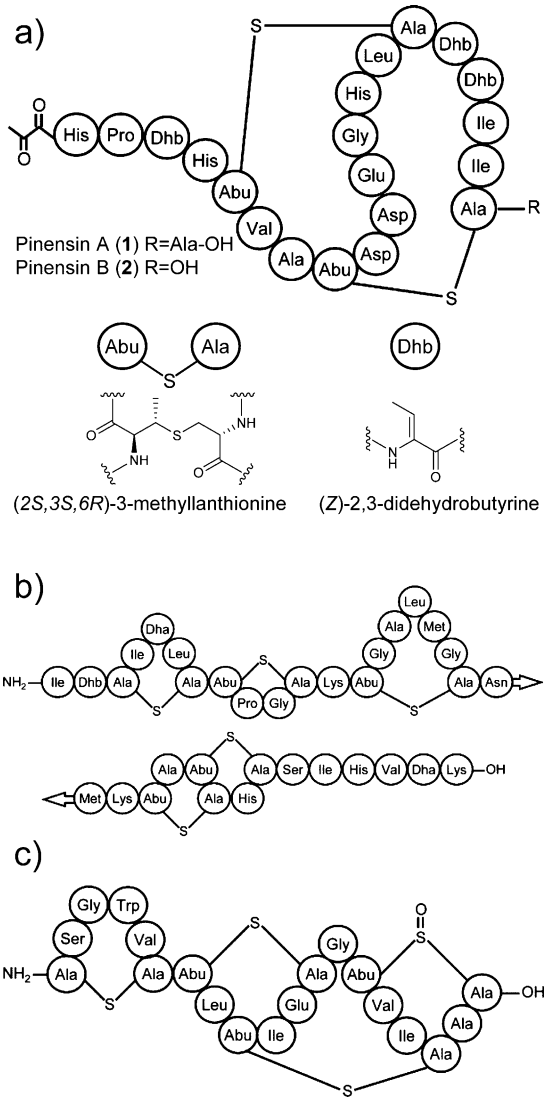

Figure 1. The structures of a) pinensins, b) nisin,[8] and c) actagardine.[9]

inhibitory concentration (MIC) of 2.1–4.2 μg mL^{-1} against yeasts and filamentous fungi.

Typical lantibiotics like nisin[8] (Figure 1b) contain three to six (methyl-)lanthionines in addition to several dehydroalanine (Dha) and dehydrobutyrine (Dhb) units.[14] Actagardine, a tetracyclic 19-residue peptide, contains one lanthionine and three overlapping 3-methyllanthionine bridges (Figure 1c).[9] However, in **1** and **2**, only two interwoven methyllanthionine rings were detected. This observation, taken together with their antifungal activity and the extraordinary genomic organization of the pinensin biosynthetic gene cluster (see below), could indicate a new (sub-)class of lantibiotics. In terms of post-translational modifications (PTMs), three Dhb units were recognized in addition to the rare pyruvic acid masking the N terminus of the peptide chain. This blocking group had been previously detected in the lantibiotics plantaricin W[18] and lactocin S[19] and was supposed to arise after cleavage of the leader sequence by rearrangement of a recruited N-terminal 2,3-didehydroalanine. Lactocin S also features only two (not interwoven) methyllanthionine rings, although it consists of 37 aa-derived residues.

Table 1: Inhibition of the growth of various cells by a 1:1 mixture of **1** and **2** (**1**/**2**) compared to reference drugs (Ref.).

Sample Type	IC_{50}[a] for **1**/**2** [μM]	IC_{50}[a] for Ref. [μM]
mouse fibroblasts L929	6.5	0.0038[d]
Huvec	2.4	0.0004[d]
KB 3.1 hela	6.5	0.0012[d]
A431 skin carcinoma	7.0	0.0006[d]
SKOV-3 ovary cancer	7.1	0.0004[d]

Sample Type	MIC[b] for **1**/**2** [μg mL^{-1}]	MIC[b] for Ref. [μg mL^{-1}]
Escherichia coli DSM 1116	n.i.[c]	1.0[e]
Mycobacterium diernhoferi DSM 43524	33.3	≤0.25[e]
Nocardia sp. DSM 43069	33.3	0.83[e]
Staphylococcus aureus DSM 346	n.i.[c]	≤0.25[e]
Chromobacterium violaceum DSM 30191	67	1.0[e]
Debaryomyces hansenii DSM 3428	33.3	6.7[f]
Pichia membranifaciens DSM 21959	4.2	0.83[f]
Rhodotorula glutinis DSM 10134	2.1	<0.25[f]
Saccharomyces cerevisiae BT27c-3 A	2.1	1.7[f]
Aspergillus clavatus DSM 816	2.1	2.1[f]
Aspergillus flavus DSM 1959*	4.2	1.0[g]
Aspergillus fumigatus DSM 15966	4.2	<0.25[g]
Botryotinia fuckeliana DSM 877	4.2	3.3[f]
Epidermophyton floccosum DSM 10709	16.6	0.25[g]
Hormoconis resinae DSM 1203	2.1	<0.25[f]
Mucor hiemalis DSM 2656	2.1	2.1[f]
Penicillium capsulatum DSM 2210	2.1	16.6[f]
Phytophthora drechsleri DSM 62679	4.2	33.3[f]

[a] MIC = minimum inhibitory concentration. [b] IC_{50} = half maximal inhibitory concentration. [c] n.i.: no inhibition. [d] epothilone A. [e] Oxytetracyclin. [f] Nystatin. [g] Amphotericin B 1.

A section of the presumed lantibiotic precursor peptide that leads to the active cleaved peptide can be predicted.[20] The D-configuration of Abu arises from L-Thr and Ala-S from L-Cys. The Dhb units arise from L-Thr. The pyruvyl group forms through 2,3-didehydroalanine from L-Ser or L-Cys. Thus, the precursor of **1** and **2** should be M[leader]S/CHPTHTVATDDQGHLCTTIIC(A). Since **1** and **2** are thought to be synthesized ribosomally, the precursor peptides should be encoded in the genome of the producer, which was published in 2010.[21] As a query, the above-mentioned aa sequence was used in a Blast[28] analysis. One ORF bearing this sequence could be found: cpin_5325 encodes a 62-aa precursor peptide of **1**. Therefore, cpin_5325 is referred to as *pinA*. No ORF could be found matching the precursor peptide of **2**, thus indicating that **2** might arise from **1** through further PTM.

The presence of the aa sequence of **1** in PinA indicates that this genomic region encodes further functions necessary for biosynthesis. Despite considerable effort, we have not yet succeeded in establishing a method for the genetic manipulation of this strain. Predictions of the biosynthesis were thus based on computational gene analysis. Nevertheless, combining knowledge about the structure and its hypothetical biosynthesis yielded first valuable insights into the biosyn-

thesis and export of this first antifungal lantibiotic produced by a Gram-negative organism.

Downstream of *pinA*, cpin_5326, cpin_5327, and cpin_5328 encode a putative LanC-like cyclase and a putative lanthionine dehydratase, respectively (Figure 3; for domain analysis see Figure S25 in the Supporting Information). The *lan* (lantibiotic) genes are *lanC*, *lanB2*, and *lanB1* according to de Vos et al.,[22] with lan substituting for a designation derived from the name of the lantibiotic. The products of these genes are likely responsible for the first two modification steps after the precursor peptide PinA is synthesized (Figure 2): PinB1 and PinB2 dehydrate amino acids. The lanthionine synthetase PinC likely forms the thioether. Interestingly, PinB1 and PinB2 might form a split LanB protein. This can be assumed based on the similarity of PinB1 and PinB2 to the NisB protein recently characterized by Ortega et al.[23] In this case, PinB1 would be responsible for aminoacylation and PinB2 would be necessary to yield the dehydroamino acid. Such split LanB proteins have been described in thiopeptide gene clusters but, to the best of our knowledge, not so far in lantibiotic gene clusters.

Cpin_5329 possesses domains necessary for export and cleavage of the modified peptides mPinA and mPinB, thus suggesting that both steps are performed by the same protein. We refer to this gene as *pinT*, according to a lantibiotic export and peptidase function of LanT. No separate peptidase function is encoded in this region. Genes *pinA* to *pinT* are considered to be the core biosynthetic cluster of pinensins.

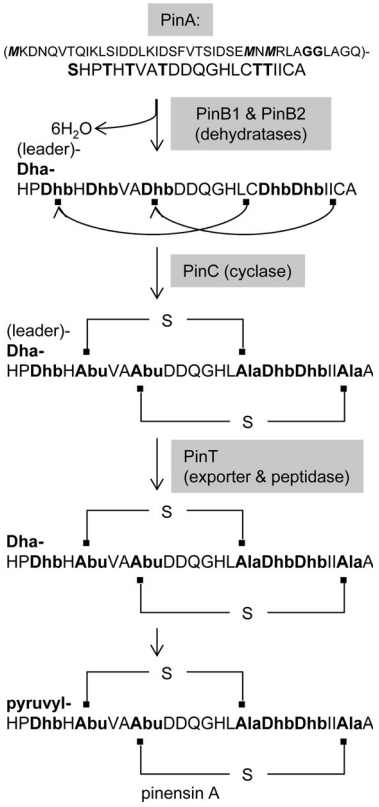

Figure 2. Amino acid sequence of PinA and proposed contributions of Pin gene products in pinensin biosynthesis. In brackets: N-terminal leader sequence of PinA. Bold italics: Putative N-terminal methionines. Bold: Putative double glycine leader site.

Based on the genetic organization, **1** and **2** can be classified as class I lantibiotics.[24,25] However, export and cleavage of the prepeptide performed by one protein (LanT) indicates a class II lantibiotic.

It is supposed that **2** arises from **1** through PTM. A respective enzymatic function therefore has to be located in the genome and might by expressed as Cpin_5319, which contains a large part of the COG5661 domain of predicted Zn-dependent proteases. The protein contains the respective HExxH motif, which is part of the metal-binding site of metalloproteases.[26] Pinensins mainly possess antifungal activity: A mechanism of self-resistance might thus not be required, which could explain the lack of any resistance determinant encoded in proximity to the *pin* region, for example, transporters or immunity proteins.[27] However, many of the genes close to the *pin* region encode alternative transport mechanisms or receptor functions (Figure 3 and Figure S25), thus providing interesting candidates to build a first overall hypothesis of pinensin biosynthesis, export, and perception. Deciphering the mechanisms of these processes would be especially intriguing because pinensins are produced by a Gram-negative bacterium but are most likely too large to pass through the outer membrane (OM). Therefore, an additional export mechanism is most likely required to cross this barrier. Based on the predicted domains present in all putative proteins encoded close to the core cluster, a prediction about the subcellular localization or function of the respective proteins can be made as follows: Activity of cytosolic PinB1, PinB2, and PinC leads to the formation of mPinA, which is exported into the periplasm by PinT and simultaneously cleaved by the same protein to yield **1**. The assumed active transport via the OM might be achieved by a TolC-dependent efflux pump encoded downstream of *pinT*. This export pump might consist of the major facilitator (MFS) transmembrane subunit Cpin_5331, which is located in the inner membrane (IM), the membrane fusion subunit Cpin_5332 (HlyD-like), and three copies of the TolC-like Cpin_5330, which is located in the OM. Whether pinensins are indeed exported by this pump must be confirmed in upcoming experiments. Intriguingly, another single MFS subunit is encoded by cpin_5318. Based on the predicted periplasmic localization of the putative enzyme involved in conversion of **1** to **2** (Cpin_5319), and the observation that **2** is present in the culture supernatant, this conversion should take place in the periplasm, thus demanding an export function to cross the OM. Finally, the TonB-dependent receptor encoded by cpin_5324 could be involved in the perception of extracellular pinensin or, together with the putative ABC transporter encoded by cpin_5321, cpin_5322, and cpin_5323, it could even be involved in import of **1** and **2** into the cytosol. Such a mechanism might be required to enable the sensing and control of extracellular pinensin levels and to enable feedback in terms of gene expression. No role could be assigned to Cpin_5320. Based on the domains present in this protein, it should be related to the IM. All of these functions are based on computational predictions. Nevertheless, these predictions, taken together with the

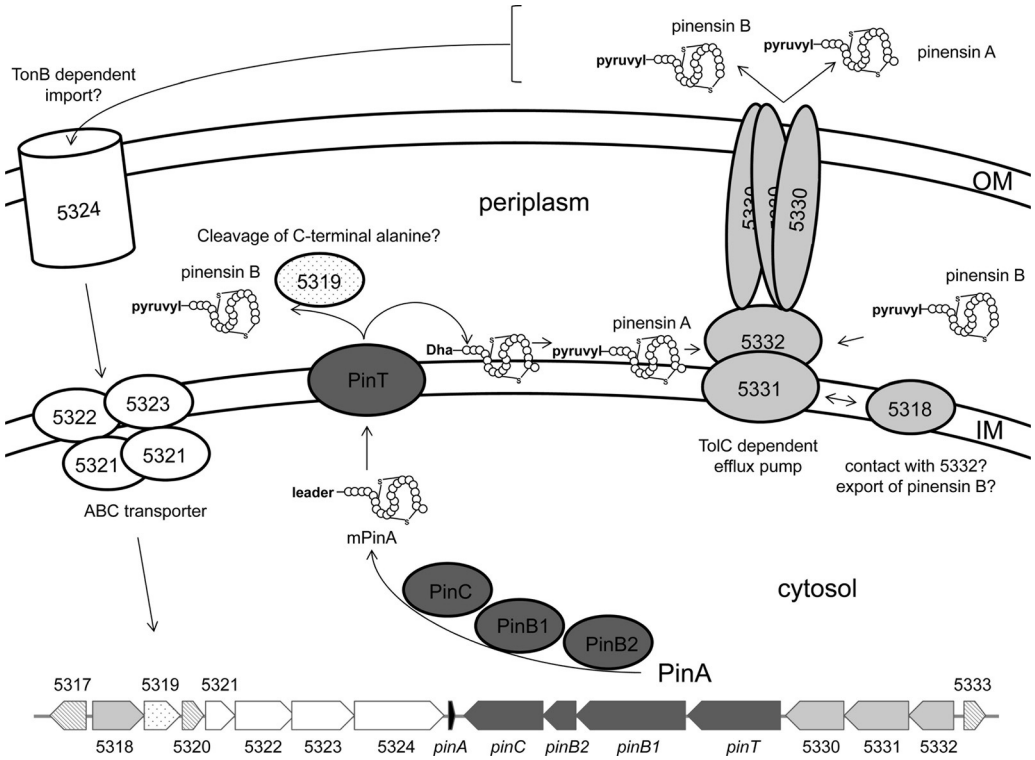

Figure 3. Hypothesis for pinensin biosynthesis and transmembrane transport. The proposed localization and function of proteins is shown in a cross-section of the cell envelope. The genomic organization of the entire assumed pinensin gene cluster is shown at the bottom. Genes are depicted as arrows. Protein/gene names are reduced to the respective numbers.

observed presence of both pinensins in the extracellular space, their production by a Gram-negative bacterium and their anti-fungal activity, as well as the fact that all of the functions described above are encoded immediately up- or downstream of the *pin* genes, strongly suggest that the boundaries of the pinensin gene cluster might be presented by the two transcriptional regulators Cpin_5317 and Cpin_5333. This hypothesis will need to be tested experimentally once a method for the transformation of *Chitinophaga* has been established.

Taking into consideration all of the data presented herein, pinensins might be regarded as an intriguing and novel (sub-)class of lantibiotics that exhibit unprecedented biological activity. Their study opens up a number of exciting opportunities for future research towards elucidating their biosynthesis and mode of action in biochemical detail, and establishing heterologous expression. Working towards these goals, we hope that the discovery of antifungal lantibiotics might eventually result in urgently needed novel therapeutic options for the treatment of fungal infections.

Keywords: biosynthesis · *Chitinophaga pinensis* · fungicides · Gram-negative bacteria · lantibiotics

How to cite: *Angew. Chem. Int. Ed.* **2015**, *54*, 11254–11258
Angew. Chem. **2015**, *127*, 11406–11410

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Received: February 9, 2015
Revised: May 26, 2015
Published online: July 24, 2015

